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Arabidopsis **STERILE APETALA**, a multifunctional gene regulating inflorescence, flower, and ovule development

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A recessive mutation in the *Arabidopsis* **STERILE APETALA** (*SAP*) causes severe aberrations in inflorescence and flower and ovule development. In *sap* flowers, sepals are carpeloid, petals are short and narrow or absent, and anthers are degenerated. Megasporogenesis, the process of meiotic divisions preceding the female gametophyte formation, is arrested in *sap* ovules during or just after the first meiotic division. More severe aberrations were observed in double mutants between *sap* and mutant alleles of the floral homeotic gene *APETALA2* (*AP2*) suggesting that both genes are involved in the initiation of female gametophyte development. Together with the organ identity gene *AGAMOUS* (*AG*) *SAP* is required for the maintenance of floral identity acting in a manner similar to *APETALA1*. In contrast to the outer two floral organs in *sap* mutant flowers, normal sepals and petals develop in *ag/sap* double mutants, indicating that *SAP* negatively regulates *AG* expression in the perianth whorls. This supposed cadastral function of *SAP* is supported by in situ hybridization experiments showing ectopic expression of *AG* in the *sap* mutant. We have cloned the *SAP* gene by transposon tagging and revealed that it encodes a novel protein with sequence motifs, that are also present in plant and animal transcription regulators. Consistent with the mutant phenotype, *SAP* is expressed in inflorescence and floral meristems, floral organ primordia, and ovules. Taken together, we propose that *SAP* belongs to a new class of transcription regulators essential for a number of processes in *Arabidopsis* flower development.

[Key Words: *Arabidopsis* mutant; inflorescence, flower, ovule development; transposon tagging; cadastral gene]

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Morphogenesis in higher plants originates from an undifferentiated group of cells, the meristem. After floral induction, the cells of a shoot apical meristem change in identity and form the inflorescence meristem that is characterized by a pattern of indeterminate growth and the production of flower meristems on its flanks. In *Arabidopsis*, these flower meristems are arranged in a spiral phyllotactic manner and are, unlike inflorescence meristems, determined. The flower meristems produce floral organ primordia in a precise number and pattern. *Arabidopsis* flowers are composed of four sepals in the first whorl, four petals in the second whorl, six stamens in the third whorl, and a pistil in the center of the flower. Inside the pistil, which is formed by the congenital fusion of two carpels, new primordia initiate and differentiate into the ovules. The initiation of primordia and the

specification of their identity require a complex network of regulatory genes. A number of these regulatory genes has been isolated from *Arabidopsis* using molecular genetic approaches (for review, see Weigel 1995). Despite the identification of these genes and extensive studies in recent years, missing pieces in the puzzle limit our understanding of the interactions between the genes involved in flower ontogeny.

The meristem identity genes *LEAFY* (*LFY*) (Weigel et al. 1992) and *APETALA1* (*AP1*) (Mandel et al. 1992) from *Arabidopsis* are involved in the establishment of the floral meristem and mutations in these two genes cause a partial conversion of a floral meristem into an inflorescence meristem. Strong *ap1* mutants produce highly branched, inflorescence-like flowers with ectopic secondary flowers in the axils of the first whorl organs (Irish and Sussex 1990; Mandel et al. 1992). *LFY* and *AP1* act synergistically together with other floral meristem identity genes, among which the *APETALA2* (*AP2*) gene (Jo-

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fuku et al. 1994). The *AP2* gene has a central role in the network of genes that control the establishment and maintenance of floral meristem and organ identity. In addition to its function as a flower meristem identity gene, *AP2* specifies whorl 1 and 2 organ identity. Furthermore, it controls the temporal and spatial expression of the homeotic gene *AGAMOUS* (*AG*) in the perianth floral whorls (Bowman et al. 1991; Drews et al. 1991) and in the ovule (Modrusan et al. 1994). Finally, aberrations in the seed coat of *ap2* mutants demonstrate that *AP2* is also required for proper seed development (Jofuku et al. 1994). The homeotic gene *AG* can also be regarded as a multifunctional gene that controls at least three critical aspects of flower development (Yanofsky et al. 1990; Bowman et al. 1991; Mizukami and Ma 1997). First, it specifies stamen and carpel identity. Secondly, it is essential for floral meristem determination, as *ag* mutants are characterized by indeterminate floral growth, reiterating the floral program inside the flower. Third, as was proposed by the ABC model (Coen and Meyerowitz 1991), *AG* negatively regulates the *AP2* gene activity in stamens and carpels. This regulation does not appear to be at the transcriptional level as *AP2* mRNA accumulation coincides with *AG* expression in the inner two floral whorls (Jofuku et al. 1994).

Several genes that are functional during floral meristem determination and flower organogenesis participate in ovule and seed development as well. Sometimes their function is masked by other genes because of redundancy. Also the *Arabidopsis* gene *SUPERMAN* (*SUP*) acts at different stages during flower development. It specifies the boundary between whorl three and four organs (Sakai et al. 1995) as well as the growth of integuments, the cell layers that surround the nucellus of the

ovule (Gaiser et al. 1995). As a final example, the *AINTEGUMENTA* (*ANT*) gene is also involved in the development of both flower organs and ovules (Elliott et al. 1996; Klucher et al. 1996).

In this report, we describe a novel *Arabidopsis* mutant, *sterile apetala* (*sap*), which is affected in inflorescence and flower and ovule development. The *SAP* gene was cloned by transposon tagging and the deduced amino acid sequence suggests that it acts most likely as a transcription regulator. Here, we demonstrate that *SAP*, in concert with *AG*, plays a role in the maintenance of floral fate in the flower. Additionally, analysis of mutant phenotypes and expression analysis revealed that *SAP* negatively controls *AG* in the inflorescence meristem and outer floral whorls. Furthermore, the observed aberrations in the ovule suggest that *SAP* is essential for the initiation of meiotic divisions during megasporogenesis. Together, these results indicate that *SAP* is a novel key regulator involved in several distinct steps of flower morphogenesis.

Results

Identification of the *sap* mutant

A large population of *Arabidopsis* plants containing the *En/Spm-1/dSpm* transposable element system (Aarts et al. 1995) was screened for mutants with reduced fertility. Plants with small or undeveloped siliques were selected. One mutant appeared to be male and female sterile as determined by reciprocal crosses to wild-type plants. This mutant (Fig. 1B) was designated *sap* because of the resemblance of its flowers to those of the homeotic *Arabidopsis* mutant *ap2* (Komaki et al. 1988; Kunst et al. 1989; Bowman et al. 1991; Jofuku et al. 1994).

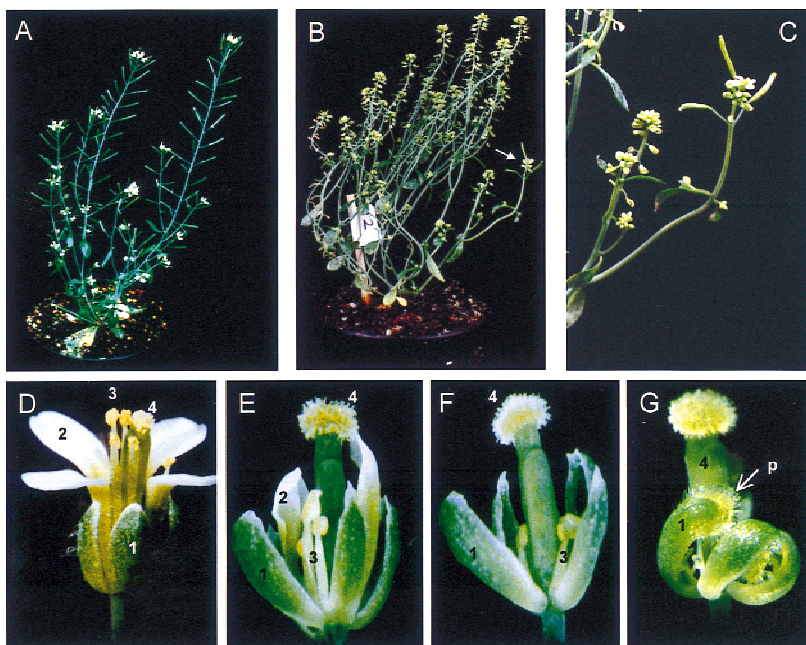


Figure 1. Phenotype of wild-type and *sap* mutant plants and flowers: (A) phenotype of a wild-type *Arabidopsis thaliana* plant. (B) Phenotype of an unstable *sap* mutant plant. Plants are sterile and internodes are shorter than in wild-type plants. Revertant branch can be observed (arrow). (C) Wild-type revertant branch (left) and a branch with a *sap* mutant phenotype (right). Reversion to wild-type phenotype occurred by excision of the transposable element from the *sap::1/dSpm* allele. (D) Phenotype of a wild-type flower. (E) Phenotype of a *sap* early-arising flower. Sepals resemble wild-type sepals. Petals are short and narrow. Stamens are short with aberrant anthers. Petals and stamens are reduced in number. (F) Phenotype of a *sap* late-arising flower. First-whorl organs are sepals with occasionally stigmatic at the tips. Petals are absent. Stamens and pistil are reminiscent with whorl -3 and -4 organs in early-arising *sap* flowers. (G) Phenotype of a severely affected late-arising flower. First-whorl organs are carpels with stigmatic papillae (p) and ovules at the inner side. Whorl-2 and -3 organs are absent.

Revertant branches were observed frequently on *sap* mutant plants producing wild-type flowers (Fig. 1C). This instability indicates that the mutation was caused by the insertion of an *I/dSpm* transposable element into the *SAP* gene, whose function was restored upon excision. Flowers from several revertant branches were self-pollinated and the progenies (35 plants each) were analyzed for *sap* mutant segregation. All progenies showed approximately a 3:1 ratio of wild-type to mutant phenotype, indicating that the parental revertant flower was heterozygous for *SAP* (*Sap,sap::I/dSpm*) and that the mutation was inherited in a Mendelian fashion as a recessive trait.

sap mutant is affected in inflorescence architecture and floral structure

In the *sap* mutant, inflorescences are produced with at least two to three times shorter internodes than in wild-type plants (Fig. 1C). Consequently, the flowers are positioned closer to each other. Mild-to-severe defects were observed in *sap* mutant flowers, depending on the age of the plant (Fig. 1E–G). The first whorl organs of *sap* flowers show a homeotic transformation from sepal to carpeloid with increasing severity in later arising flowers. Petals in the second whorl of the early-arising flowers are narrower and shorter than in wild-type flowers. In later-arising flowers the petals are completely absent and the four first-whorl organs are transformed into carpels bearing ovules at the inner side (Fig. 1G). These aberrations in the outer two whorls of *sap* flowers resemble those observed in the flowers of the *Arabidopsis* mutant *ap2* (Komaki et al. 1988; Kunst et al. 1989; Jofuku et al. 1994). The number of stamens in the third whorl of *sap* flowers is reduced from six to four (missing the lateral stamens) or two. These stamens have shorter and thinner filaments and small degenerated anthers, which do not produce pollen. A detailed microscopic analysis showed that the tissues in the anthers did not differentiate normally (results not shown). The fourth whorl of a *sap* flower consists of a morphologically normal pistil with stigmatic papillae, which are longer than in wild-type plants.

Ovule development is abnormal in sap mutants

Flowers from *sap* plants are female sterile and never produce seeds upon pollination with wild-type pollen. To investigate whether female sterility is caused by defects in pollen–pistil interaction, we followed the pollen tube growth in *sap* pistils. Pollen grains were able to germinate on the stigma and the pollen tubes grew inside the pistil towards the ovary, with the formation of regular callose plugs as can be observed in wild type (Fig. 2A). However, penetration of pollen tubes into *sap* ovules was abolished, suggesting that the ovules were aberrant (Fig. 2B,C). Failure of pollen tube penetration was also observed by Hülkamp et al. (1995) in the *Arabidopsis* female sterile mutants *bell1*, *short integuments* (*sin1*), *47H4*, and *54D12*.

Surprisingly large accumulation of callose was observed in mature *sap* ovules. To study this abnormal callose deposition and the ontogeny of *sap* ovules in more detail, we performed a microscopic analysis on ovules of various developmental stages. Detailed analyses of ovule development in *Arabidopsis* have been reported previously (Robinson-Beers et al. 1992; Modrusan et al. 1994; Schneitz et al. 1995). In wild-type *Arabidopsis* ovules at stage 9 (Smyth et al. 1990), a megasporocyte is differentiated in the nucellus (Fig. 2D). Shortly afterward, at stage 10, when the formation of the two integuments is initiated, the megasporocyte undergoes two cycles of meiotic divisions resulting in four megaspores (megasporogenesis, Fig. 2E). Only one out of the four megaspores is functional and survives and forms, after three cycles of nuclear division and cellularization (megagametogenesis), a mature embryo sac (Fig. 2F). During megagametogenesis, both integuments continue to grow and cover the nucellus completely by late stage 12.

Ovule development in the *sap* mutant is indistinguishable from wild type at early stages before meiosis (Fig. 2G). All sporophytic tissues, such as nucellus, inner and outer integuments, and funiculus develop normally (Fig. 2H,I,L,M). The first aberrations became apparent during megasporogenesis, because a tetrad was never observed in *sap* ovules (Fig. 2H). In mature *sap* ovules, embryo sacs are absent completely and callose accumulates at the position normally occupied by the embryo sac (Fig. 2C,I).

To analyze the megasporogenesis in wild-type and in *sap* ovules in more detail, aniline blue staining was performed to visualize callose in newly formed cell walls (Rodkiewicz 1970; Schneitz et al. 1995). Figure 2J shows an ovule at the dyad and one at the tetrad stage in a wild-type ovary. In *sap* ovules, the megasporocyte undergoes the first meiotic division, but does not continue cell division. Frequently, callose does not only accumulate in the cell wall of the dyads but deposition was also observed inside the cells (Fig. 2K). Accumulation of callose inside megaspores is characteristic for cells that degenerate (Pimienta and Polito 1982; Vishnyakova 1991), which probably occurs in *sap* ovules at or immediately after the first meiotic division. At later stages, when the integuments extend towards the apex of the nucellus, still only two megaspores are present and they are filled with callose (data not shown). In mature *sap* ovules, callose accumulation is observed at the position where in wild-type ovules an embryo sac is formed (Fig. 2C).

Molecular cloning of the SAP gene

For the cloning of the *SAP* gene, the *I/dSpm* transposable element that cosegregates with the *sap* phenotype was identified. DNA flanking this *I/dSpm* element was amplified by an inverse polymerase chain reaction (IPCR) (Aarts 1996) and used as a probe for the screening of a wild-type *Arabidopsis thaliana* Landsberg *erecta* genomic library. A 9-kb genomic fragment was isolated. The structure of this genomic fragment and the location of

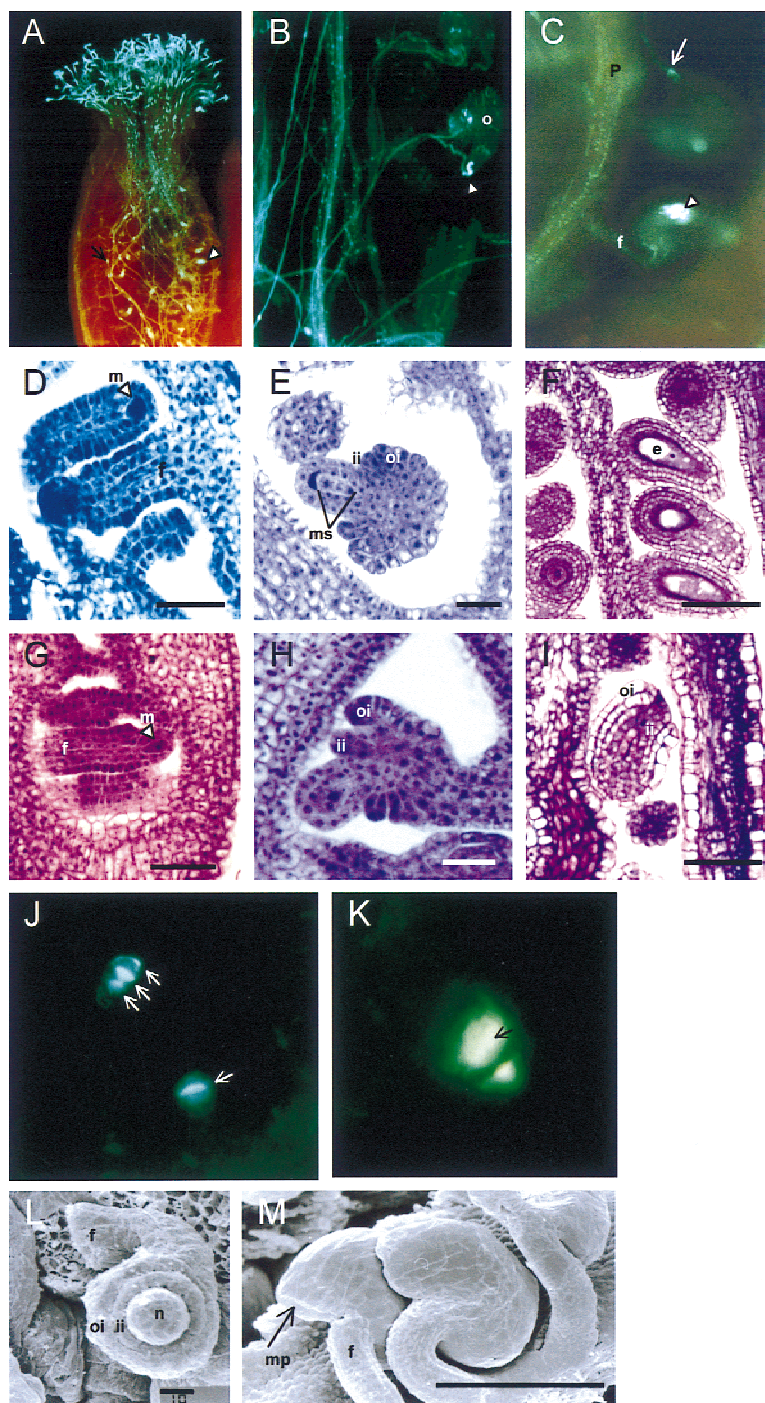


Figure 2. Microscopic analysis of wild-type and *sap* mutant ovules. (A) UV micrograph showing pollen tube growth through a mature pistil of *sap*. Pollen tubes are visualized by aniline blue. Regular callose plugs inside the pollen tubes (arrow) and strong staining in ovules (arrowhead) can be seen. (B) UV micrograph showing the penetration of pollen tubes into wild-type ovules. The arrowhead points to a successful penetration into the micropyle of the ovule. (C) UV micrograph of two ovules in a pollinated *sap* mutant plant. The upper ovule is morphologically normal and is penetrated by a pollen tube (arrow). Wild-type ovules appeared rarely in the *sap* mutant. They are most likely caused by somatic excision of the transposable element from the *SAP* gene during early stages of ovule development. The lower ovule has a typical *sap* phenotype with a highly stained plug of callose in the center of the ovule. An arrowhead indicates this callose deposition. (D) Light micrograph of developing wild-type ovules at stage 9 (Smyth et al. 1990). The megasporocyte is indicated by an arrowhead. (E) Light micrograph of developing wild-type ovule at stage 10 (Smyth et al. 1990). The four megaspores are formed in the nucellus after two cycles of meiotic divisions. Simultaneously, the outer and inner integuments appear. (F) Light micrograph of mature wild-type ovules. Outer and inner integuments surround embryo sacs. (G) Light micrograph of developing *sap* ovules at stage 9 (Smyth et al. 1990). The megasporocyte is indicated by an arrowhead. (H) Light micrograph of developing *sap* ovule at stage 10 (Smyth et al. 1990). Megasporogenesis is arrested and only two megaspores can be detected. (I) Light micrograph of a mature *sap* ovule. Embryo sac is absent, but outer and inner integuments are indistinguishable from wild-type integuments. (J) and (K) UV micrographs of aniline blue stained ovules of wild-type plants (J) and *sap* mutant plants (K). (J) A tetrad and dyad are visible in the wild-type ovules. Callose deposition is indicated by an arrow. (K) Stage 10 (Smyth et al. 1990) of a *sap* ovule with an aberrant dyad. Callose accumulates inside the megaspores as indicated by an arrow. (L) and (M) Scanning electron micrographs of stage 10 *sap* ovules (L) and mature *sap* ovules (M). The sporophytic tissues develop normally and the integuments enclose the nucellus completely. (o) Ovule; (m) megasporocyte; (ms) megaspores; (oi) outer integument; (ii) inner integument; (f) funiculus; (e) embryo sac; (p) placenta; (mp) micropyle. Bar, 10 μ m (D,E,G,H,L); 50 μ m (F,I) 100 μ m (M).

the *I/dSpm* element insertion in *SAP* are illustrated in Figure 3A.

To prove that the insertion in the cloned fragment cosegregates with the *sap* mutant phenotype, a blot containing DNA of a segregating *Sap/sap* progeny was hybridized with a part of the isolated fragment as probe. All *sap* mutant plants (6 plants) were homozygous for the specific *I/dSpm* element insertion, whereas all plants (21 plants) with a wild-type phenotype exhibited either two

hybridizing fragments or only one band representing the wild-type allele (data not shown).

Excision of an *I/dSpm* element often leads to footprints in the form of short DNA deletions or additions (Aarts et al. 1995). Therefore, a few stable *sap* mutants and *Sap* revertant plants with a wild-type phenotype were chosen for transposon-footprint analysis. The DNA sequences of the derived alleles are shown in Figure 3C. The stable *sap* plant (*SAP-5.510*) carries a 4-bp addition,

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dicted molecular mass of 49 kD. The coding region is followed by a 3' untranslated region of 390 bp. Based on the comparison of the genomic and cDNA sequence, it is concluded that the *SAP* gene contains a single intron of ~3 kb. This was confirmed by comparison of fragments obtained by RT-PCR and PCR on genomic DNA using *SAP*-specific primers (data not shown).

A computer search of the available sequence databases using the BLAST program (Altschul et al. 1990) revealed no significant similarities of the predicted *SAP* protein sequence to known protein sequences. When we compared the *SAP* cDNA sequence with the EST (expressed sequence tag) database of *Arabidopsis*, no clear homologies were detected either. However, a further examination of the *SAP* protein revealed small sequence motifs that are characteristic for transcriptional regulators. A long serine-rich stretch (STSSSSS) is present at the amino terminus of the protein. Similar domains have been found in a number of eukaryotic transcription factors and it has been implicated that these sites are targets for phosphorylation (Mitchell and Tjian 1989). This amino-terminal serine-rich region is adjacent to a glycine-rich region (DNGAGSGG). Together these two stretches compose a serine/glycine-rich domain, which is a motif often found in eukaryotic transcriptional regulators (Janssen-Timmen et al. 1989; Gashler et al. 1993). In conclusion, *SAP* encodes a novel protein containing a few hallmarks of a transcriptional regulator.

SAP is expressed predominantly in inflorescence and floral meristems and ovules

Phenotypic analysis of *sap* mutant plants indicates that the *SAP* gene product is required for inflorescence and flower and ovule development. To determine how the expression pattern of *SAP* correlates with the *sap* mutant phenotype, we studied the *SAP* mRNA distribution in wild-type *Arabidopsis* plants by in situ hybridization.

SAP transcripts first become detectable in the inflorescence meristem, and *SAP* remains uniformly expressed in the floral meristems throughout stages 1 and 2 of flower development (Fig. 4A,B). After stage 3, when sepal primordia have started to arise, *SAP* expression becomes restricted to floral whorls 2, 3, and 4, whereas no hybridizing signal was detected in whorl 1 (Fig. 4B). Expression is maintained at approximately the same level in developing petals, stamens, and carpel primordia until stage 6. After stage 6, when the organ primordia emerge further, the level of *SAP* expression diminishes and no *SAP* mRNA is detectable at stage 8 (Fig. 4C). At later developmental stages *SAP* transcript appears again in ovule primordia in the nucellus region before the initiation of integuments. When integument primordia start to arise (stage 10), the expression of *SAP* increases and is predominantly detectable in the developing inner and outer integuments (Fig. 4F). In expanding integuments, *SAP* expression seems to be maintained at the same level, but it decreases at mid-stage 12 and becomes restricted to the basal part of the integuments. No expression was detected in mature ovules (data not shown).

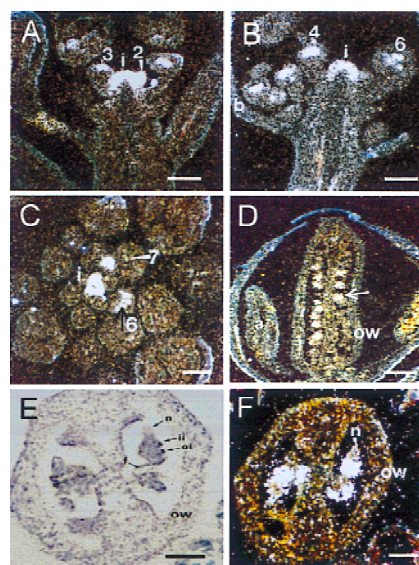


Figure 4. Expression of *SAP* mRNA in wild-type Landsberg *erecta* inflorescence and flowers. Numbers refer to the stage of flower development according to Smyth et al. (1990). Expression of *SAP* was determined by in situ hybridization with 35 S-labeled antisense RNA corresponding to the *SAP* cDNA. Hybridization signals were viewed using dark-field microscopy. (i) Inflorescence meristem; (b) bract; (p) petal; (a) anther; (s) stigma; (ow) ovary wall; (oi) outer integuments; (ii) inner integument; (f) funiculus; (n) nucellus. (A,B) Longitudinal sections through inflorescence. Strong expression is detected in the inflorescence meristem and at the flanks of the apex, corresponding to the initiation of a floral primordium and young floral buds (stages 1 and 2). At flower stages 3–4 expression is detectable in the central region between the sepal primordia. At stage 6, hybridization signals are visible in the primordia of the petals, the stamens and the central gynoecium but not in the sepals. (C) Cross section through an inflorescence apex. The developing floral buds surround the inflorescence meristem in a spiral pattern. Expression is maintained throughout stages 2–5 and decreases during stage 6, whereas expression is abolished after stage 7. (D) Longitudinal section of a stage-12 flower bud. Strong *SAP* expression is present in ovules (arrow), but not in other floral organs. (E,F) Cross sections through a pistil of stage 11 imaged under bright field (E) or dark-field (F). Strong hybridization signals are present in the developing outer and inner integuments, and weaker signals are detectable in the nucellus. Bars, 100 μ m (A–D); 50 μ m (E,F).

The in situ hybridization experiments demonstrate that the location of *SAP* expression in wild-type *Arabidopsis* plants is correlated with the phenotypic aberrations observed in the *sap* mutant. The homeotic transformations in the first whorl of *sap* flowers appear to be initiated at a very early stage, because at later developmental stages when sepal primordia arise, *SAP* expression is not detectable anymore.

To determine whether residual *SAP* mRNA is present in *sap* mutant plants, Northern blot analysis was performed using poly(A)⁺ RNA isolated from inflorescences with young flower buds at various developmental stages (0–6). Although the expression of *SAP* is weak, accumulation of *SAP* mRNA was detectable in wild-type inflo-

rescences, but was abolished completely in inflorescences of the *sap* mutant (data not shown). Because there is a strong resemblance between the phenotypes of *sap* and the *ap2* mutant, we reprobated the Northern blot with *AP2* to analyze whether the *sap* phenotype was partly caused by altered *AP2* gene expression. However, *AP2* is expressed in the *sap* mutant at the same level as in wild-type inflorescences (data not shown). A reciprocal Northern blot carrying mRNA from three *ap2* mutant alleles (*ap2-1*, *ap2-5*, *ap2-6*), and probed with *SAP*, did not show difference in expression either (data not shown), indicating that there is no direct correlation between *AP2* and *SAP* expression.

Interaction between *SAP* and homeotic genes

Some features of the *sap* mutant resemble characteristics of *ap2* mutants, which may suggest a possible genetic interaction between these two genes. To test this hypothesis we produced and analyzed double-mutant plants. Because it was demonstrated previously (Drews et al. 1991) that *AP2* is a negative regulator of *AG*, we also raised double mutants between *sap* mutant plants and the *ag-1* mutant allele.

sap/ap2 double mutants

Several *ap2* mutant alleles were used to raise double mutants with *sap*. We used the weak *ap2-1* allele in our double-mutant studies to monitor a possible synergistic effect of *SAP* in an *ap2* mutant background. In *ap2-1* flowers, leaves develop in whorl 1, a reduced number of staminoid petals are formed in the second whorl, and normal stamens and carpels develop in whorls 3 and 4, respectively (Bowman et al. 1991, Fig. 5A). As is shown in Figure 5B, *sap/ap2-1* flowers possess features of both *sap* and *ap2-1* single mutants. Whorl one organs are leaf-like as in *ap2-1* single mutants implying that *AP2* is epistatic to *SAP* in the first whorl. With respect to whorl two organs, the genes seem to act synergistically because *sap/ap2-1* double mutants never produce second whorl organs. The morphology of the stamens resembles those of the *sap* single mutant. The pistil is normal morphologically though the size is somewhat reduced, resulting in a smaller number of ovules.

Like *sap* single mutants *sap/ap2-1* plants are completely sterile. Defects in ovule development appear to be enhanced in *sap/ap2-1* mutants. Microscopic analysis (Fig. 5, cf. C and D) reveals that *sap/ap2-1* ovules not only lack an embryo sac, as in *sap* single mutants, but in addition the outer integument does not cover the inner integument and nucellus completely. This strong synergistic effect suggests that both genes are essential for normal outer integument development, in accordance with the expression patterns of *SAP* and *AP2* (Jofuku et al. 1994).

The *sap* single mutant shows defects in megasporogenesis, therefore we examined whole mount of *sap/ap2-1* ovules to visualize callose deposition during cell

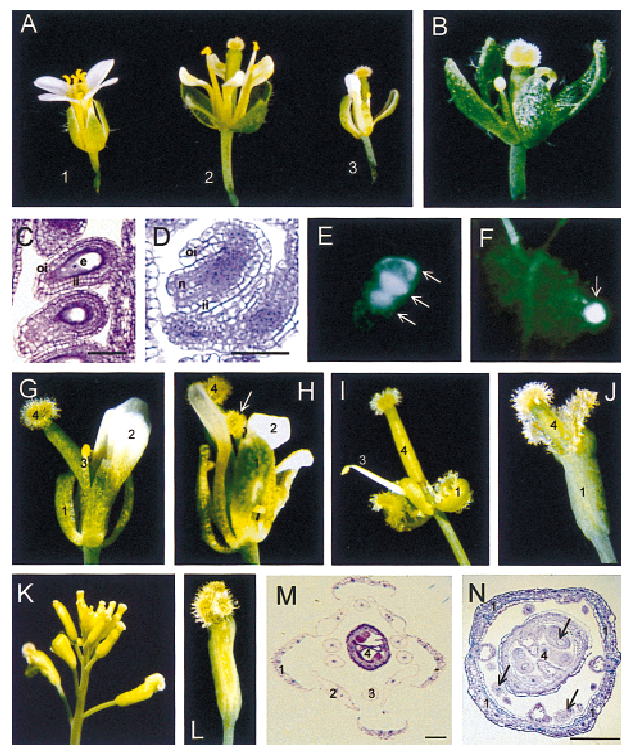


Figure 5. Phenotypes of *sap/ap2* double mutants. (A) Phenotypes of wild-type (1), *ap2-1* (2), and *sap* (3) mutant flowers. (B) Phenotype of *sap/ap2-1* double mutant flower. Sepals are leaf-like as in *ap2-1* single mutants and petals are completely absent. The number of stamens is reduced and anthers are malformed or carpeloid. Pistil is smaller than in *ap2-1* and *sap* single mutants. (C) Longitudinal section through mature wild-type ovules. Outer and inner integuments surround the embryo sacs. (D) Longitudinal section through a mature *sap/ap2-1* ovule. These ovules exhibit protruding nucellus (n) and the outer integument (oi) does not enclose the ovule completely. Bar, 50 μ m. (E) UV micrograph of cleared wild-type ovule at stage 9 (Smyth et al. 1990) stained with aniline blue to visualize callose deposition. A tetrad is visible and the arrows indicate callose deposition. (F) UV micrograph of a cleared *sap/ap2-1* ovule at stage 9 (Smyth et al. 1990) stained with aniline blue to visualize callose deposition. Callose accumulates (arrow) in the nucellus before megaspore formation. (G) Phenotype of an early-arising *sap* flower. (H) Phenotype of an *ap2-5* mutant flower. First whorl organs show carpeloid features with occasionally stigmatic tissue (arrow). Petals are reduced in size. Stamens are reduced in number but develop normally. The pistil is not affected. (I) Phenotype of an *ap2-6* mutant flower. Whorl-one organs are transformed into carpels, petals are absent and the number of stamens is reduced. (J) Phenotype of an early-arising *ap2-5/sap* double mutant flower. Four carpels are formed in whorl one, which are partially fused to a gynoeceum. Petals and stamens are completely absent. The pistil contains ovules with similar aberrations as is observed in *sap/ap2-1* double mutants. (K) Inflorescence of an *ap2-5/sap* double mutant. (L) Phenotype of a late-arising *ap2-5/sap* double mutant flower. Four completely fused carpels are formed in whorl one, which covers the central gynoeceum. (M) Light micrograph of a cross section through a wild-type flower. The floral whorls are indicated. The central gynoeceum is composed of two fused carpels. (N) Light micrograph of a cross section through a *sap/ap2-5* flower. The four carpels in whorl 1 are fused to a tube. Ovules in the outer and inner gynoeceum are indicated with arrows. Bars, M and N, 100 μ m.

division. In these ovules, callose becomes apparent as a group of intensely fluorescing cells at the chalazal region of the nucellus at stage 9 (Fig. 5, cf. E and F), when differentiation of a megasporocyte occurs in wild-type plants. In contrast to *sap* single mutants, megaspores, either as dyad or tetrad, were never observed in *sap/ap2-1* ovules, indicating that mutations in both genes lead to an arrest in megasporogenesis at earlier stages than in *sap* single mutants. The fact that *ap2-1* ovules display a wild-type phenotype indicates that *AP2* is redundant during megasporogenesis and acts in a different pathway to control ovule development other than *SAP*.

We also raised double mutants between *sap* and stronger alleles of *ap2*. The *ap2-5* mutant allele (Kunst et al. 1989) has carpelloid sepals, which produce occasionally stigmatic tissue (Fig. 5H). The petals are reduced in size and the number of fertile stamens is reduced to four or two. No morphological abnormalities are observed in *ap2-5* pistils. The *ap2-6* (Kunst et al. 1989) is a strong allele with whorl-one organs transformed into unfused carpels and petals completely missing. The reproductive organs are fertile, although the number of stamens is reduced dramatically (Fig. 5I). *sap/ap2-5* and *sap/ap2-6* double mutants are very similar indicating that the maximum synergistic level is reached (Fig. 5J–L). In both types of double mutants, the four sepals are transformed completely into carpels, which are fused to a gynoeceum-like structure. In side this structure, only the whorl-four pistil is present, whereas petals and stamens are absent. Cross sections through these flowers show that the outer pistil is composed of four carpels bearing ovules at the inner side (Fig. 5M,N). Analysis of the ovules from these double mutants using aniline blue staining for callose deposition revealed a similar abnormality as shown in Figure 5F for the *sap/ap2-1* double mutant. In conclusion, these double-mutant studies demonstrate that the *sap* mutant is synergistic to *ap2* mutant alleles.

sap/ag-1 double mutants

Double mutants between *sap* and the *ag-1* mutant allele were generated and displayed a remarkable phenotype (Fig. 6A,D–F). Early-arising *sap/ag-1* flowers resemble *ag-1* single mutants exhibiting an indeterminate number of whorls of sepals and petals (Fig. 6B,C). The first-whorl organs are not carpel-like as in *sap* flowers but more sepaloid implying that *AG* is epistatic to *SAP* with respect to first-whorl organ identity. The petals in the second whorl of the double mutant are shorter and narrower than the petals in both *ag-1* and wild-type flowers (Fig. 6D). In contrast to the *sap* mutant the number of organs in the second and third whorl are as in wild-type flowers. Later-arising *sap ag-1* flowers (from approximately seventh flower) produce secondary flower buds in the axils of the second and all subsequent floral whorl organs (Fig. 6D–F). This pattern of floral bud formation reiterates in axillary flowers giving rise to a highly branched inflorescence-like structure (Fig. 6F). When the axillary flowers develop, the regions between the floral whorls elongate forming floral internodes in such a way that the floral

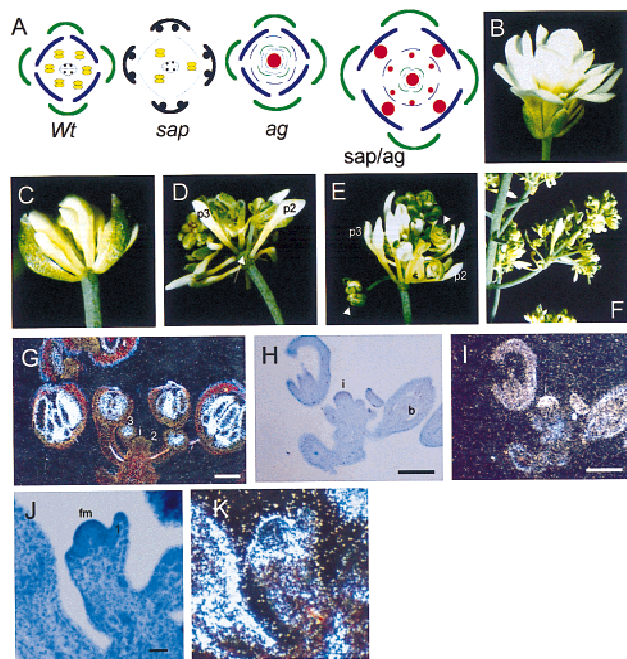


Figure 6. Phenotypes of *sap/ag* double mutants and expression pattern of *AG* in wild-type and *sap* inflorescences. (A) Schematic representation of position and identity of the floral organs in wild-type (wt), *sap*, *ag-1*, and *sap/ag-1* mutant flowers. Organs are marked as follows: sepals (green); petals (blue); degenerated petals in *sap* (thin blue lines); stamens (yellow); carpels with ovules (black); new floral buds (red). (B) Phenotype of an *ag-1* mutant flower. (C) Phenotype of an early-arising *sap/ag-1* double mutant flower. One sepal was removed to show the inner floral organs. *sap/ag-1* flowers are indeterminate with a repeated pattern of sepals-petals-petals from the outermost whorl to the center as in *ag-1* single mutants. Compared to wild-type petals, petals of *sap/ag-1* are shorter and narrower as in *sap*. (D–E) Developing *sap/ag-1* flowers resembling inflorescences. Secondary flowers, indicated by an arrowhead, develop in axils of the second (p2) and third (p3) whorl petals and all internal floral organs. Internodes are formed between the floral whorls (arrow). (F) Old *sap/ag-1* flower producing an inflorescence-like branch. (G–K) Expression of *AG* was determined by in situ hybridization with ^{35}S -labeled antisense RNA. Hybridization signals were viewed using dark-field microscopy. (i) Inflorescence meristem; (b) bud; (fm) floral meristem. (G) Distribution of *AG* mRNA in a wild-type inflorescence. Signal appears in the center of buds at early stage 3 and in older buds only in stamens and carpels. (H–I) Longitudinal section through a *sap* inflorescence imaged under bright field (H) or dark field (I). Strong hybridizing signal is visible in the inflorescence meristem and in the entire bud (b). These buds are composed of only carpelloid sepals and a gynoeceum in the fourth whorl. (J–K) Longitudinal section through a *sap* floral meristem at early stage 3 of flower development imaged under bright field (J) or dark field (K). Strong hybridizing signal is visible in a ring around the floral apex. The first-whorl primordia just emerge at the flanks of the meristem.

organs and secondary flowers are arranged in a spiral phyllotaxis rather than a whorled mode as in wild type. These indeterminate flowers display several features of an inflorescence, such as elongated internodes and spiral phyllotaxis. This can be interpreted as a partial reversion from floral to an inflorescence identity.

The phenotype of *sap/ag-1* double mutants demonstrates that characteristics of the *sap* mutant depend on the presence of an active *AG* gene, suggesting that *SAP* repress the expression of *AG* in the outer two floral whorls. To investigate this in more detail, the distribution of *AG* mRNA in *sap* flowers was analyzed. In wild-type flowers, *AG* is not expressed in inflorescence meristems and stage 1–2 floral buds (Fig. 6G). At early stage 3, when the sepal primordia just emerge, *AG* mRNA is first detectable in the floral apex and not in the sepal primordia. At later developmental stages, hybridization signals were observed in stamen and carpel primordia only. In contrast, high expression levels were detectable in inflorescence meristems of the *sap* mutant. *AG* mRNA is also present in all whorls of the highly affected floral buds, which are composed of carpelloid sepals and a developing gynoecium (Fig. 6H,I). In *sap* flower buds at a stage when the first-whorl organs just emerge at the flanks of the floral meristem (Fig. 6J,K), hybridization signal is detectable in a ring around the floral apex and at a lower level in the first whorl primordia and apex. These data indicate that, unlike wild-type flowers, *AG* is expressed in inflorescence meristems and at the earliest stages of *sap* flower formation.

Discussion

Upon floral induction, the inflorescence meristem normally produces flowers with whorls of distinct organs. We have demonstrated that the *Arabidopsis* *SAP* gene, consistent with its mRNA expression pattern, is involved in several floral developmental programs. In concert with *AG* it is required to maintain floral meristem identity, it controls floral organ identity by suppressing *AG* activity in the outer whorls and it interacts with *AP2* to initiate megasporogenesis in ovules. Sequence comparison revealed that the *SAP* gene encodes a novel protein with no substantial sequence similarities to other known proteins. However, small domains inside the *SAP* protein share sequences present in transcriptional regulators, suggesting that *SAP* may play a role in controlling several developmental programs at the transcriptional level.

SAP is required for megasporogenesis

The *sap* mutant is female sterile, which trait is inherited in a sporophytic manner. Microscopic analysis of the development of *sap* ovules revealed no obvious aberrations in the ontogeny of the sporophytic tissues of the female reproductive organ, whereas megasporogenesis is arrested after the first meiotic division. These observations imply that *SAP*, as a sporophytically controlled gene, is involved directly in female gametophyte development and presumably plays a crucial role during meiotic cell division. No information is available about the role of *SAP* in microsporogenesis, because anthers degenerate prematurely in *sap* mutant plants.

Several mutants that affect female gametophyte devel-

opment in *Arabidopsis* have been described (for review, see Schneitz et al. 1997), though the majority of these mutants display aberrations in sporophytic as well as gametophytic tissues of the ovule. Often, defects in integument development are accompanied by partial or complete absence of megasporogenesis, as it is observed in the *sin1*, *ant* and *huellos* (*hll*) mutants (Robinson-Beers et al. 1992; Lang et al. 1994; Elliott et al. 1996; Klucher et al. 1996; Schneitz et al. 1997). These mutants are referred to as sporophytic and megagametogenesis-defective (*smd*) mutants by Schneitz et al. (1997). Another class of female defective mutants, from which only a few members have been described (Schneitz et al. 1997) are characterized by defects in gametogenesis and are designated embryo sac-defective (*emd*) mutants. According to this classification, the *sap* mutant belongs to the class of megasporogenesis-defective (*msd*) mutants, from which only *SAP* has been cloned. The *emd* and *msd* mutants provide valuable knowledge about the interdependency between sporophytic and gametophytic tissues. Based on the mutants, we conclude that defects in sporophytic tissues often lead to aberrant gametophyte development, however, in contrast sporophyte development is independent from megasporogenesis and megagametogenesis.

SAP genetically interacts with *AP2* in various developmental programs

The *SAP* gene is strongly expressed in the inflorescence meristem and the emerging floral primordia in wild-type *Arabidopsis* plants. This pattern of expression overlaps with that of *AP2* and it also coincides with *AP2* expression at later developmental stages, during floral organogenesis and ovule development. Combined with the resemblance of the phenotypes of the single mutants, we hypothesized that *SAP* and *AP2* interact genetically. Expression analysis revealed that *AP2* expression is not affected in the *sap* mutant and, vice versa, demonstrating that they do not regulate each other at the transcriptional level.

Although both genes are expressed in the inflorescence meristem, the phenotype of the single mutants revealed that only *SAP* is essential for establishing the inflorescence structure. The complete transformation of sepals into carpels and the absence of petals in the *ap2-5/sap* and *ap2-6/sap* double mutants indicate that the genes are synergistic.

Another intriguing aspect of the interaction between *SAP* and *AP2* is the more dramatic defect observed during ovule development. Megasporogenesis in all double mutants analyzed appears to be arrested at even an earlier stage than in the *sap* single mutant, whereas the *ap2* mutant alleles are normally fertile. This new phenotype indicates that both genes *SAP* and *AP2* are involved in the initiation of megasporogenesis and are able to complement each other at this stage. This ascribes a new function for *AP2*, which is normally masked by redundancy of genes also involved in this process. Because in *sap* ovules the development proceeds until the second

meiotic division, *SAP* is dispensable for the initial stage of megasporogenesis but absolutely required for later processes. The fact that the aberrations in the *sap* mutant are inherited as a recessive trait indicates that *SAP* is a sporophytically expressed gene controlling the reduction to haploid spore cells.

SAP genetically interacts with AG to maintain floral meristem identity

sap/ag-1 double mutants display several interesting transformations with respect to floral architecture as well as meristem identities. First, the restoration of the first-whorl organ identity to sepals and the formation of petals in the second and third whorls demonstrate that the role of *SAP* in these whorls depends on *AG* expression.

A second feature of the double mutant is the formation of axillary flower buds in the axils of the organs in the second and all subsequent whorls. The organs in these whorls and the axillary flowers are arranged in a spiral phyllotaxy, which is typical for inflorescences. Similar inflorescence-like characteristics and reversions to inflorescence shoot meristem are present in *ap1* and *lfy* single and *ag/ap1*, *ag/lfy* double mutants (Bowman et al. 1993; Weigel et al. 1992; Schultz and Haughn 1991). Even in *ag* single mutants grown under short day conditions, reversions of the indeterminate floral meristem to the indeterminate inflorescence meristem occurs independently from the early acting genes *AP1* and *LFY* (Mizukami and Ma 1997). Because single *sap* and *ag* mutant flowers grown under our conditions do not exhibit transformations into inflorescence-like structures, the phenotype of the double mutant indicates that *SAP* acts locally in concert with *AG* either to maintain or to promote floral meristem fate. The residual floral character of the transformed flowers is revealed by the presence of sepal and petal-like organs and implies that *SAP* and *AG* interact in a local manner similar to *AP1*. Most likely, the early acting meristem identity genes *LFY* and *AP1* initiate a determinate floral meristem, whereas several other genes are required for the final definition of floral meristem identity. Here, we demonstrated that at least two genes, *SAP* and *AG*, facilitate in part the final definition of floral meristem identity in *Arabidopsis*.

SAP is a negative regulator of AG

One of the functions of *AP2* is to prevent ectopic expression of the *AG* gene in the perianth whorls, but *AP2* seems not to be capable of suppressing *AG* on its own (Drews et al. 1991; Okamuro et al. 1993; Jofuku et al. 1994). Recently, it was shown that two other genes, *LEUNIG* (*LUG*) and *CURLY LEAF* (*CLF*) may play a role in concert with *AP2* to repress *AG* in the first- and second-whorl organs of *Arabidopsis* flowers (Liu and Meyerowitz 1995; Goodrich et al. 1997). *lug* mutants are characterized by narrow leaves and floral organs and display an enhanced *ap2* phenotype with respect to floral-

organ identity. Mutations in *CLF* cause severe effects on leaf morphology and on flowering time. In addition, the flowers of *clf* are very similar to *ap2* flowers, though the petals are smaller and narrower compared to petals of weak *ap2* alleles. Double-mutant analysis and misexpression of *AG* in *ap2*, *lug*, and *clf* mutants have demonstrated that these genes act as negative regulators of *AG* at different stages of development (Drews et al. 1991; Liu and Meyerowitz 1991; Goodrich et al. 1997). *AP2* and *LUG* are both required for the initial specification of *AG* expression pattern from stage 2 onwards. In *clf* mutants, *AG* is expressed ectopically in hypocotyls, cotyledons, leaves, and at later floral stages (from stage 9) in the petals. This suggests that *CLF* is needed to maintain the suppression at later developmental stages.

Several observations provide evidence that *SAP*, like *AP2*, *LUG*, and *CLF*, is a cadastral gene negatively regulating *AG* expression. First, the phenotype of *sap* single and *ap2/sap* double mutants show striking similarities to that of plants in which *AG* was constitutively expressed (Mizukami and Ma 1992). The double mutants exhibit more severe homeotic transformation in flowers than either single mutant. This corroborates our suggestion that *SAP* is one of the partners of *AP2* to suppress *AG*. Secondly, in *ag/sap* double mutants sepals and petals develop, which demonstrates that for *SAP* action in the perianth organs *AG* expression is needed. Furthermore, it shows that *SAP* is not a floral organ identity gene required to specify sepal and petal identity. Finally, our in situ hybridization experiments reveal that *AG* is misexpressed in the inflorescence meristem and during the initial stages of *sap* floral organ development. We did not detect *SAP* expression in sepal primordia and in flower buds after developmental stage 7, suggesting that another regulator causes the carpelloidly in the first whorl of *sap* mutants. Alternatively, the carpelloidly is already established before the sepal primordia emerge (before stage 2), when *SAP* mRNA is present and *AG* is ectopically expressed in a *sap* mutant background. The role of *SAP* in specifying the spatial expression of *AG* is additive to that of the other cadastral genes *AP2*, *LUG*, and *CLF*, because none of these regulators control *AG* in the inflorescence meristem.

This report and earlier observations with *AP2*, *LUG*, and *CLF*, demonstrates that a complex pattern of spatially restricted genes, each acting at different developmental stages is required to co-ordinatively regulate the expression of the homeotic gene *AG*.

Materials and methods

Plant material

A. thaliana ecotype Landsberg *erecta* was used in this work. All plants were grown under standard greenhouse conditions (16 hr light; 20–22 °C). T₃ seeds of two *En/Spm-I/dSpm* transposon-containing lines were used for the screening for sterile mutants (Aarts et al. 1995). The original *sap::I/dSpm* transposon-tagged mutant was found in the line H32.3 containing ~10 different *I/dSpm* elements.

Seeds of *ag-1* and *ap2* mutant alleles were obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH).

Construction of double mutants

sap/ap2 As *sap* mutant plants are completely sterile we pollinated *Sap,sap::1/dSpm* heterozygous flowers with *ap2* pollen. F_1 progeny of those crosses were all phenotypically normal. Seeds obtained from a few self-fertilized F_1 plants were harvested and the progenies were analyzed. As the *ap2-5* and *ap2-6* mutants were in the Columbia genetic background the appearance of the F_2 plants varied slightly with respect to size and internode length depending on the genetic background. Among the F_2 progeny four flower phenotypes were observed consistent with a 9:3:3:1 ratio, as listed below. For *sap/ap2-1*, the distribution of F_2 plants was 173 wild-type, 51 *ap2-1*, 54 *sap*, and 18 plants with a novel phenotype (ratio 9:3:3:1; $\chi^2 = 0.013$; $P > 0.9$). For *sap/ap2-5*, the distribution of F_2 plants was 71 wild-type, 21 *ap2-5*, 19 *sap*, and 8 plants with a novel phenotype (ratio 9:3:3:1; $\chi^2 = 0.75$; $P > 0.9$). For *sap/ap2-6*, the distribution of F_2 plants was 57 wild type, 20 *ap2-5*, 16 *sap*, and 6 plants with a novel phenotype (ratio 9:3:3:1; $\chi^2 = 0.5$; $P > 0.9$). Some of the plants with the novel phenotype produced revertant branches with a normal length of the internodes (in F_2 *sap/ap2-1*) and with fertile *ap2* flowers indicating that they represented *sap/ap2* double mutants.

sap/ag-1 To obtain *sap/ag-1* double mutants stable, heterozygous *Sap,sap::1/dSpm* plants were crossed with the *Ag,ag-1/Sap,sap::1/dSpm* self-pollinated plants four different phenotypes were found represented by 159 wild-type, 55 *ag-1*, 50 *sap*, and 17 plants with a novel phenotype (ratio 9:3:3:1; $\chi^2 = 0.26$; $P > 0.90$). A few plants with the novel phenotype had revertant branches with *ag-1*-like flowers, indicating that the new phenotype belonged to *sap/ag-1* double mutants.

Inverse PCR analysis

DNA flanking the *I/dSpm* element was obtained by IPCR as described by Aarts (1996). The resulting IPCR product was cloned in pMOSBlueT-vector (Amersham) and sequenced with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer).

Mapping of the SAP locus

For the mapping of *SAP*, a PCR fragment obtained by using the *SAP* gene-specific primer 1 (5'-TCGACTCATTTAAGGCTTC-3') and primer 2 (5'-CAGTGCACCGAAATCCCATAGATG-3'), was used as RFLP probe. This probe was hybridized to a blot containing the *Sst*I-digested DNAs of 98 RILs (Lister and Dean 1993). Recombination frequencies and map distances were calculated with the JoinMap program (Stam 1993), using mapping data provided by C. Lister and C. Dean (Cambridge Laboratory, John Innes Institute, Norwich, UK).

Genomic library screening

A Landsberg *erecta* genomic library, obtained through the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus, OH) and the European DNA Resource Centre (Max-Delbrück Laboratory, Köln, Germany), was screened with the *SAP*-specific IPCR fragment as a probe. Hybridizations were done as described previously (Angenent et al. 1992).

DNA and RNA analysis

Genomic DNA from *Arabidopsis* leaves was isolated as described by Aarts (1996), and 400 ng was digested, electrophorized, and blotted onto Hybond N+ membranes. Blots were hybridized according to Angenent et al. (1992) and washed with $0.1\times$ SSC at 65°C.

Poly(A)⁺ RNA from different *Arabidopsis* tissues was isolated using a QuickPrep Micro mRNA Purification Kit as described by manufacturer (Pharmacia Biotech), and 5 μ g was denaturated by glyoxal (1.5 M) prior to electrophoresis. Hybridization was performed according to Angenent et al. (1992).

In situ hybridization

The plant material was fixed at 40°C for 2 days in 4% formaldehyde, 5% acetic acid, 50% ethanol, and stepwise dehydrated until 100% ethanol prior to infiltration with xylene and subsequent embedding in paraffin and sectioning. Sections treated with paraffin and rehydrated were taken according to the in situ hybridization procedure essentially as described by Angerer and Angerer (1991). The complete *SAP* cDNA was used for generating the antisense probe. The *AG* antisense RNA contained nucleotides 240–977 of the *AG* sequence published in Yanofsky et al. (1990). Hybridization was done at 42°C in $2.25\times$ SSPE ($1\times$ SSPE: 0.18 M NaCl, 0.001 M NaPO₄ at pH 7.7, 0.001 M EDTA), 50% formamide, and washed at 42°C in $0.1\times$ SSC, 50% formamide. Sections were exposed for 2–4 weeks (*AG* probe) and 8 weeks (*SAP* probe). Photographs were taken with a Diaplan microscope equipped with dark-field optics (Leitz, Wetzlar, Germany).

5' RACE

5' RACE was performed according to the protocol of the Marathon cDNA Amplification Kit (Clontech). Briefly, 1 μ g of polyadenylated RNA from young flower buds (stages 0–6) was used as a template for first-strand cDNA synthesis. The first PCR was performed using adaptor primer 1 and the *SAP* gene-specific primer 3 (5'-GTGTGACCAACACCGTCTATTTC-3'), which corresponds to nucleotides 713–739 of *SAP* cDNA. A second round of PCR was done using adaptor primer 2 and a nested gene-specific primer 4 (5'-ATCCGTCGGCGAACCCTCCG-3'), corresponding to nucleotides 537–555 of *SAP* cDNA. The resulting 5'-RACE-PCR product of 555 bp in length was cloned and sequenced as described before. The accession number of the *SAP* cDNA sequence is AJ223125.

Callose and pollen tube staining in ovaries

Pollen tube growth and callose deposition were visualized by staining with aniline blue as described previously (Angenent et al. 1993).

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